Volume 317, number 1,2, 74–78 FEBS 12069 February 1993

Human amnion epithelial cells assemble tenascins and three fibronectin isoforms in the extracellular matrix

Auli Linnala[a], Enrica Balza[b], Luciano Zardi[b] and Ismo Virtanen[a]

[a]*Department of Anatomy, University of Helsinki, Siltavuorenpenger 20 A, 00170 Helsinki, Finland* and [b]*Laboratory of Cell Biology, Istituto Nazionale per la Ricerca sul Cancro, Viale Benedetto XV, 10, 16132 Genoa, Italy*

Received 9 December 1992

Monoclonal antibodies (MAb) were used to show that cultured human amnion epithelial (HuA) cells produce tenascins (Tn) and isoforms of cellular fibronectin (cFn). Tn polypeptides of M_r 280,000 and 190,000, assembled into extracellular matrix (ECM) but not secreted into the culture medium by HuA cells, were electrophoretically similar to those produced by human fibroblasts as revealed with domain-specific MAbs. The results suggested that most Fn produced by HuA cells contained the extradomain (ED) A and an oncofetal domain but only a minor fraction EDB. In immunofluorescence Tn and Fn were seen in different cytoplasmic granules upon monensin-induced intracellular accumulation. Tn appeared to be deposited in the ECM in colocalization with Fn but distinctly slower. The present results show that cultured normal human epithelial cells synthesize Tn and three isoforms of cFn and secrete them by using different cytoplasmic pathways.

Amnion epithelial cell; Fibronectin; Tenascin; Extracellular matrix; Secretion

1. INTRODUCTION

Tn is an extracellular matrix protein sharing sequence homology with Fn, epidermal growth factor and fibrinogen [1–4] and has been considered to function in various epithelial–mesenchymal interactions during development [2,3,5,6] and to become prominently expressed in stromal areas of carcinomas [2,5,7] and healing wounds [8]. Immunohistochemical results have attributed synthesis of Tn to mesenchymal cells [3,5,6] and in a recent review epithelial cells were suggested to be unable to produce Tn [2]. In recent in situ hybridization studies, however, transcripts of Tn were found in several developing epithelial cells [9–11] some of them considered earlier unable to produce Tn (cf. [12]).

In the present study we have studied the production of Tn and isoforms of cFn in cultures of HuA cells, earlier found to produce Fn, laminin and collagens [13]. The present results show that the HuA cells produce Tn and also assemble the protein into extracellular matrix together with three distinct, alternatively spliced forms of cFn.

2. MATERIALS AND METHODS

2.1. *Cell culture*

HuA cells were obtained by trypsinization of dissected amniotic membranes as described earlier (cf. [14]) and cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS; Gibco, Paisley, Scotland) and antibiotics. As revealed by IF with cytokeratin MAbs the cultures were free of fibroblastic contamination (not shown; see [14]). Human embryonal skin fibroblasts were obtained from a local source and were cultured as above. In some experiments the cells were exposed to monensin (1 μM; Eli Lily, Indianapolis, IN) for 16 h to prevent cellular secretion [15,16]. For immunoprecipitation experiments with the MAbs to different Fn isoforms and gelatin-Sepharose (Pharmacia, Uppsala, Sweden) affinity chromatography [17] spent culture medium of HuA cells was collected. For some experiments the serum-free culture media were exposed to $(NH_4)_2SO_4$ precipitation at 4°C overnight. Metabolic labeling with [^{35}S]methionine (Amersham, Buckinghamshire, UK; spec. act. 1,000 mCi/mol) was performed by using an overnight incubation (20 μCi/ml) of the cells in MEM medium, lacking methionine and supplemented with dialysed 1% FCS. To produce cell-free ECM, 0.5% sodium desoxycholate (DOC) in 10 μM Tris-HCl (pH 7.4) was used according to Hedman et al. [18] after 2 weeks in primary culture or approx. one week after first trypsinization. DOC-matrices of HuA cells and human fibroblasts were then subjected to immunoblotting and immunoprecipitation experiments.

2.2. *SDS-PAGE, immunoblotting and fluorography*

The MAbs 100EB2 [7], BC-2 [4] and BC-4 [4] against human tenascin, DH1 against EDAcFn [19], BC-1 reacting with EDBcFn [20,21] and FDC-6 reacting with an oncofetal epitope in type III connecting segment region of cFn [22] have been characterized earlier. For immunoblotting of DOC-matrices, $(NH_4)_2SO_4$ precipitates of the culture medium, polypeptides bound to gelatin-Sepharose from HuA cells and fibroblasts and immunoprecipitates of the HuA cell culture medium the samples were boiled in the electrophoresis sample buffer [23] and run in 6.5% slab gels. The polypeptides were transferred onto nitrocellulose according to Towbin et al. [24] and immunostained with the MAbs by using the indirect immunoperoxidase method. For immunoprecipitation of metabolically labeled DOC-matrices the specimens were dissolved in 1% Triton X-100, 1% DOC and 0.1% SDS in 50 mM Tris-HCl (pH 7.4). After centrifugation the supernatant was exposed to the MAbs and the antigen–antibody complexes were recovered by using agarose-bound rabbit anti-mouse Igs (Cappel, Organon-Teknika, West Chester, PA) and subjected to SDS-PAGE. The samples were then subjected to fluorography.

Correspondence address: A. Linnala, Department of Anatomy, University of Helsinki, Siltavuorenpenger 20 A, SF-00170 Helsinki, Finland. Fax: (358) (0) 191 8499.

 Published by Elsevier Science Publishers B.V.

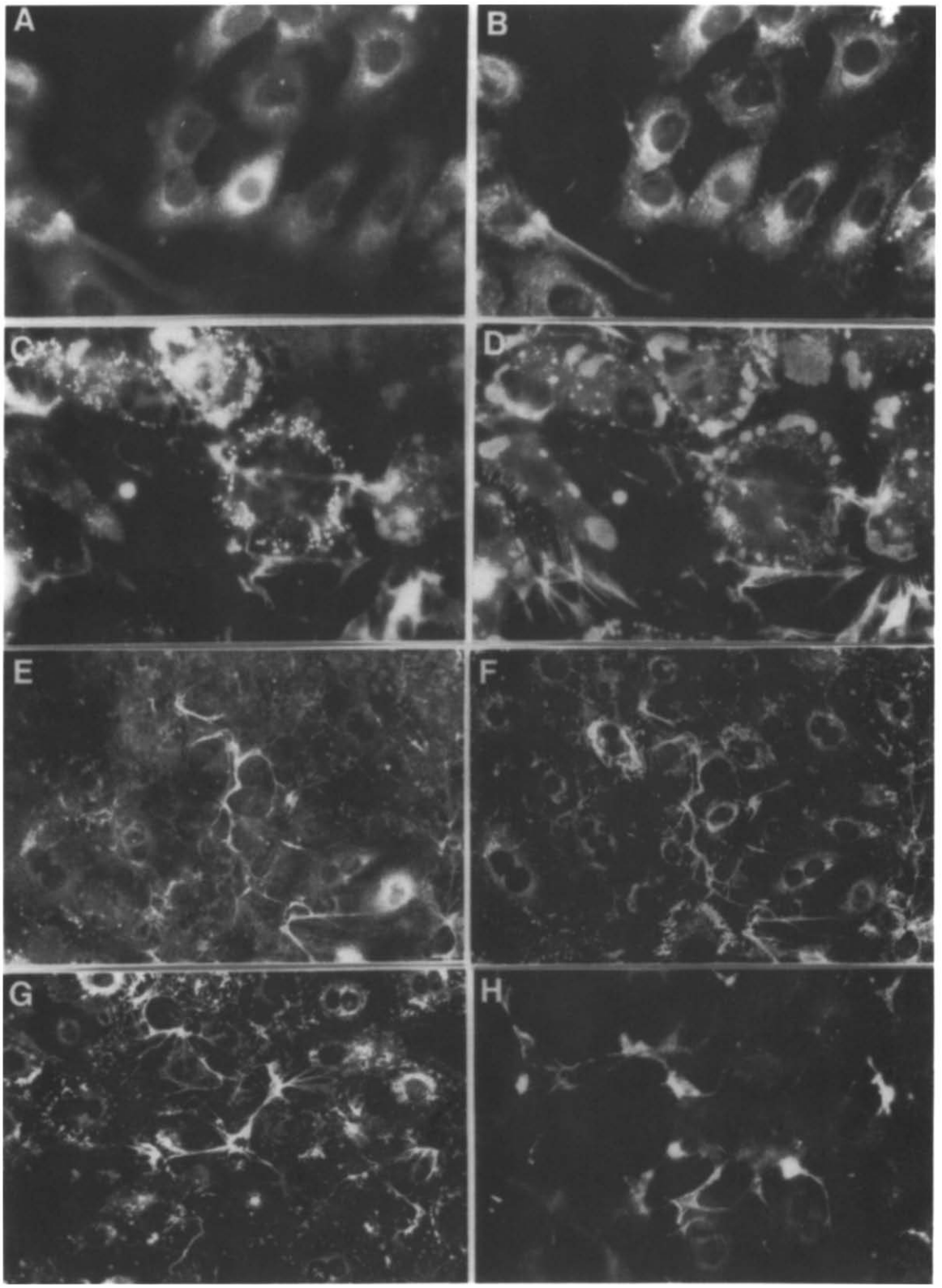

Fig. 1. Double IF of HuA cells for Tn (A) and Fn (B) reveals a bright cytoplasmic reactivity for Tn in only some of the cells while all cells present Fn-reactivity. Double IF of monensin-exposed cells revealed Tn in small cytoplasmic granules (C) while Fn was present in larger vacuoles (D). In 8-day cultures both Tn (E) and Fn (F) are seen in ECM fibrils. MAbs reveal in IF EDAcFn (G) and EDBcFn (H). Magnification ×600.

2.3. *Indirect immunofluorescence (IF) microscopy*

For IF the HuA cells were cultured on small coverslips and fixed in methanol, cooled to −20°C. Then the cells were reacted with the MAbs for 30 min. Fluorescein isothiocyanate (FITC)-coupled sheep anti-mouse IgG antiserum (Cappel) was then applied.

For double IF the cells were first exposed to rabbit anti-Fn antiserum (Dakopatts, Glostrup, Denmark) followed by FITC-coupled sheep anti-rabbit IgG (Cappel), then to the Mab 100EB2 followed by tetramethylrhodamine isothiocyanate (TRITC)-coupled sheep anti-mouse IgG (Cappel). The specimens were examined in a Leitz Aristoplan microscope equipped with appropriate filters.

3. RESULTS

To study the ECM polypeptides synthesized by HuA cells we first analysed the spent culture medium produced by the cells; repeated immunoprecipitation experiments of the radioactively labeled culture medium or immunoblotting experiments of the $(NH_4)_2SO_4$ precipitates of the culture medium failed to reveal Tn in HuA cell cultures (not shown). However, double immunostaining for Tn (Fig. 1A) and Fn (Fig. 1B) of freshly seeded HuA cells revealed a cytoplasmic Tn immunoreactivity in codistribution with Fn in many but not in all of the cells. Exposure of such cultures to monensin induced an accumulation of Tn into small cytoplasmic granules (Fig. 1C) while Fn immunoreactivity was seen in distinctly larger vacuoles (Fig. 1D; see also [19]). In 8 day cultures an emerging codistribution of Tn immunoreactivity (Fig. 1E) with extracellular Fn fibrils (Fig. 1F) was seen. Immunostainings for EDA- (Fig. 1G), EDB- (Fig. 1H) and onc-cFns (not shown) revealed that all forms of the protein are deposited into ECM fibrils by the HuA cells.

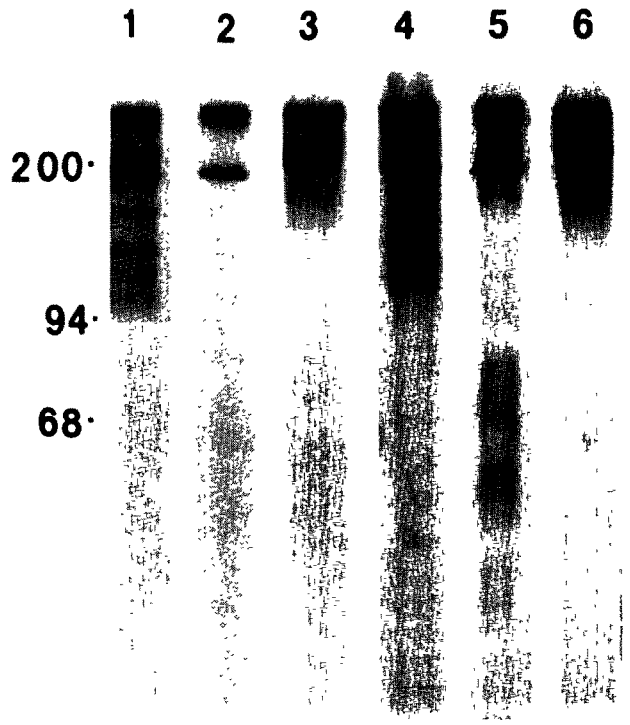

Fig. 2. Immunoblotting of SDS-PAGE separated polypeptides of cell-free ECM of HuA cells (lanes 1–3) and cultured human fibroblasts (lanes 4–6). The MAb 100EB2 reveals polypeptides of M_r 280,000, 210,000 and 190,000 in both cells (lanes 1 and 4), the MAb BC-4 the M_r 280,000 and 190,000 (lanes 2 and 5) while the MAb BC-2 reveals only the M_r 280,000 and diffuse M_r 210,000 bands (lanes 3 and 6). The M_r of the control proteins is indicated on the left.

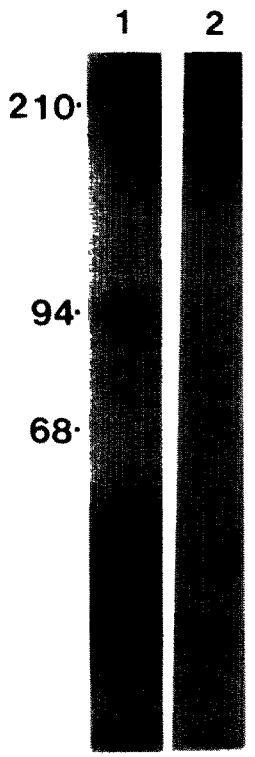

Fig. 3. Immunoprecipitation of cell-free ECMs of metabolically labeled HuA cells with the MAbs 100EB2 (lane 1) and DH1 (lane 2). Fluorography reveals polypeptides on M_r 280,000 and 190,000 in lane 1 and a major polypeptide of M_r ca. 240,000 in lane 2. The M_r of control proteins is indicated on the left.

To study further Tn polypeptides, the cell-free ECM of HuA cells were produced by the DOC-treatment. Immunoblotting experiments with the MAb 100EB2 to Tn revealed two distinct polypeptides of M_r 280,000 and 190,000 and a diffuse band of ca. M_r 210,000 (Fig. 2, lane 1), identical to those found in ECM of cultured fibroblasts (Fig. 2, lane 4). Immunoblotting with the MAb BC-4, that reacts with an epitope in the aminoterminal part of the molecule, detecting all Tn polypeptides [4], revealed the same major polypeptide doublet (Fig. 2, lane 2) while the MAb BC-2, reacting with an epitope within the FN-like repeat which undergoes alternative splicing in the 280 kDa Tn [4], revealed the M_r 280,000 and diffuse ca. M_r 210,000 bands (Fig. 2, lane 3). Similar polypeptides were revealed in cell-free ECM of human fibroblasts (Fig. 2, lanes 4–6).

Immunoprecipitation experiments of metabolically labeled whole cells (not shown) and DOC-matrices revealed a doublet of Tn polypeptides of M_r 280,000 and

190,000 (Fig. 3, lane 1) differing from those precipitated by the MAb DH1 to EDAcFn (Fig. 3, lane 2).

Immunoblotting experiments with MAbs to Fn isoforms of gelatin-Sepharose-bound polypeptides from the culture medium of HuA cells revealed that seemingly equivalent amounts of EDAcFn (Fig. 4, lane 1) and onc-cFn (Fig. 4, lane 3) were bound, but significantly less EDBcFn (Fig. 4, lane 2). Further immunoprecipitation experiments of unlabeled HuA culture medium subjected to immunoblotting after SDS-PAGE with the MAb DH1 revealed that the EDAcFn immunoprecipitate contained equivalently EDA and the oncofetal domain but appeared to lack EDB (Fig. 5, lanes 1–3).

4. DISCUSSION

Fn is known to be an adhesive glycoprotein playing role in many dynamic phenomena during development, wound healing and tumorigenesis [25] while at least in vitro Tn appears to interfere with Fn function and may have opposing effects also in vivo [2,26,27]. Earlier immunohistochemical and expression studies of Fn isoforms, with thusfar unknown functions, suggested their differential regulation in cultured cell lines [21,28],

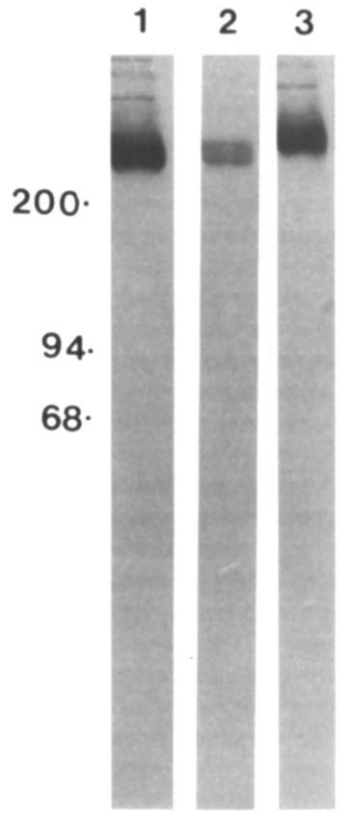

Fig. 4. Immunoblotting of gelatin-Sepharose-bound polypeptides from the culture medium of HuA cells with the MAbs DH1 (lane 1), BC-1 (lane 2) and FDC-6 (lane 3). Note, that similar prominent polypeptides of M_r 240,000 are seen in lanes 1 and 3 while less reactivity for EDBcFn is seen (lane 2).

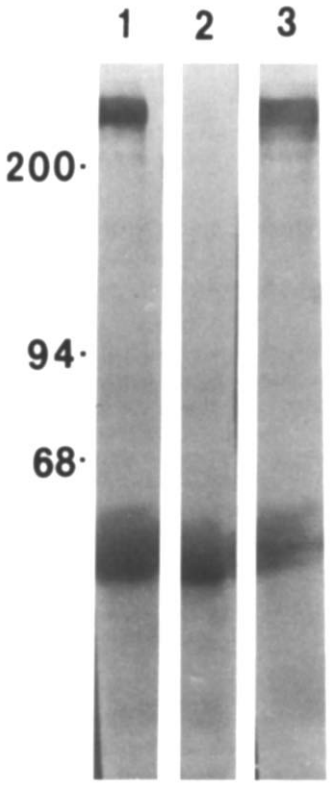

Fig. 5. Immunoblotting with cFn MAbs of polypeptides immunoprecipitated from the HuA cell culture medium by the MAb DH1 to EDAcFn. Immunoreactivities for EDAcFn (lane 1) and onc-cFn (lane 3) appear similar while no reactivity is seen for EDBcFn (lane 2).

during development (e.g. [29,30]) and malignant change [2,7]. While in situ hybridization studies on developing tissues have suggested that also epithelial cells express Fn isoforms [30] contrasting results on human epithelial tumors have been reported [31,32].

Our results with isoform-specific MAbs revealed that the HuA cells produced cFn containing EDA, EDB and an oncofetal domain. Earlier studies on cultured mesenchymal cells have suggested that the percentage of cFn molecules from malignant and transformed cells would contain higher amounts of EDB and oncofetal domain [21,22,28]. The present results suggest that in HuA cells most cFn molecules contain both EDA and oncofetal domain while a minor portion of the molecules would contain only the EDB.

The HuA cells appeared to produce Tn polypeptides of M_r 280,000- and 190,000-like normal human fibroblasts [7] and were also found in vivo [3,7]. This could be shown also with domain-specific MAbs that gave identical blotting results with both cell types. Results with the MAb BC-4 suggest that the third diffuse M_r 210,000 band represents degradation products which have lost the aminoterminal part of the high M_r Tn. Earlier studies [33] have suggested that the low M_r Tn polypeptide would mostly participate in ECM assem-

bly. Our results show, however, that both in human epithelial and fibroblastic cells both Tn polypeptides are similarly present in the ECM.

Interestingly enough, while cFns could be easily detected in the culture medium and ECM, Tn could only be found in the ECM of HuA cells. This would suggest that Tn and Fn are differently secreted, Tn in a polarized manner to the underlying ECM and Fn randomly to the medium. This was supported by the finding that monensin induced cessation of secretion, and subsequent intracellular accumulation of secretory products [15,16] revealed cFn and Tn in distinctly different intracellular vacuoles. Epithelial cells are generally able to traffic intracellular protein transport in a polarized way (e.g. [34]). Recently, Wang et al. [35] were able to show that transforming growth factor β regulates the polarized secretion of EDAcFn in tracheal epithelial cells but thusfar only endothelial cells have been shown to be able to secrete ECM-associated proteins via different intracellular pathways [36].

Tn production has been mostly attributed to mesenchymal cells. These present results show that attention should be paid also to the function of Tn in epithelia.

Acknowledgements: The MAb FDC-6 was kindly provided by Prof. S.-i. Hakomori (Seattle, WA). The skillful technical assistance of Mrs. Pipsa Kaipainen, Mrs. Anita Mikkola and Mrs. Marja-Leena Piironen is acknowledged. The study was supported by the Finnish Medical Research Council, the Sigrid Juselius Foundation, the University of Helsinki and by funds from Associazione Italiana per la Ricerca sul Cancro and Consiglio Nazionale per la Ricerche 'Progetto fina Pizzato: Applicazioni cliniche della ricerca oncologica'.

REFERENCES

[1] Nies, D.E., Hemesath, T.J., Kim, J.-H., Gulcher, J.R. and Stefansson, K. (1991) J. Biol. Chem. 266, 2818–2823.
[2] Chiquet-Ehrismann, R. (1990) FASEB J. 4, 2598–2604.
[3] Weller, A., Beck, S. and Ekblom, P. (1991) J. Cell. Biol. 112, 355–362.
[4] Siri, A., Carnemolla, B., Saginati, M., Leprini, A., Casari, G., Baralle, F. and Zardi, L. (1991) Nucleic Acids Res. 19, 525–531.
[5] Chiquet-Ehrismann, R., Mackie, E.J., Pearson, C.A. and Sakakura, T. (1986) Cell 47, 131–139.
[6] Aufderheide, E., Chiquet-Ehrismann, R. and Ekblom, P. (1987) J. Cell. Biol. 105, 599–608.
[7] Howeedy, A.A., Virtanen, I., Laitinen, L., Gould, N.S., Koukoulis, G.K. and Gould, V.E. (1990) Am. J. Pathol. 63, 798–806.
[8] Mackie, E.J., Halfter, W. and Liverani, D., (1988) J. Cell Biol. 107, 2757–2767.
[9] Onda, H., Poulin, M.L., Tassava, R.A. and Chiu, I.-M. (1991) Dev. Biol. 148, 219–232.
[10] Prieto, A.L., Jones, F.S., Cunningham, B.A., Crossin, K.L. and Edelman, G.M. (1990) J. Cell Biol. 111, 685–698.
[11] Koch, M., Wehrle-Haller, B., Baumgartner, S., Spring, J., Brubacher, D. and Chiquet, M. (1991) Exp. Cell Res. 194, 297–300.
[12] Mackie, E.J., Halfter, W. and Livcrani, D. (1988) J. Cell Biol. 107, 2757–2767.
[13] Alitalo, K., Kurkinen, M., Vaheri, A., Krieg, T. and Timpl, R. (1980) Cell 19, 1053–1062.
[14] Regauer, S., Franke, W.W. and Virtanen, I. (1985) J. Cell Biol. 100, 997–1009.
[15] Tartakoff, A.M. (1980) Int. Rev. Exp. Pathol. 22, 227–251.
[16] Virtanen, I., Vartio, T., Badley, R.A. and Lehto, V.-P. (1982) Nature 298, 660–663.
[17] Engvall, E. and Ruoslahti, E. (1977) Int. J. Cancer 20, 1-5.
[18] Hedman, K., Kurkinen, M., Alitalo, K., Vaheri, A., Johansson, S. and Höök, M. (1979) J. Cell Biol. 81, 83–91.
[19] Vartio, T., Laitinen, L., Närvänen, O., Cutolo, M., Thornell, L.-E., Zardi, L. and Virtanen, I. (1987) J. Cell Sci. 88, 419–430.
[20] Carnemolla, B., Leprini, A., Allemanni, G., Saginati, M and Zardi, L. (1992) J. Biol. Chem. in press.
[21] Carnemolla, B., Balza, E., Siri, A., Zardi, L., Nicotra, M.R., Bigotti, A. and Natali, P.G. (1989) J. Cell Biol. 108, 1139–1148.
[22] Matsuura, H., Takio, K., Titani, K., Greene, T., Levery, S.B., Salyan, M.E.K. and Hakomori, S.-I. (1988) J. Biol. Chem. 263, 3314–3322.
[23] Laemmli, V.K. (1970) Nature 227, 680–685.
[24] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[25] Ruoslahti, E. (1988) Annu. Rev. Biochem. 57, 375–414.
[26] Chiquet-Ehrismann, R., Kalla, P., Pearson, C A., Beck, K. and Chiquet, M. (1988) Cell 53, 383–390.
[27] Mackie, E.I., Thesleff, I. and Chiquet-Ehrismann, R. (1987) J Cell Biol. 105, 2569–2579.
[28] Borsi, L., Balza, E., Allemanni, G. and Zardi, L. (1992) Exp. Cell Res. 199, 90–105.
[29] Laitinen, L., Vartio, T. and Virtanen, I. (1991) Lab. Invest. 64, 492–498.
[30] ffrench-Constant, C. and Hynes, R.O. (1989) Development 106, 375–388.
[31] Moro, L., Colombi, M., Molinari Tosatti, M.P. and Barlati, S. (1992) Int. J. Cancer 51, 692–697.
[32] Peltonen, J., Jaakkola, S., Lask, G., Virtanen, I. and Vitto, J. (1988) J. Invest. Dermatol. 91, 289–293.
[33] Chiquet-Ehrismann, R., Matsuoka, Y., Hofer, U., Spring, J., Bernasconi, C. and Chiquet, M. (1991) Cell Regul. 2, 927–938.
[34] Wellner, D.A. and Nelson, W.J. (1992) J. Cell Sci. 102, 185–190.
[35] Wang, A., Cohen, D.S., Palmer, E. and Sheppard, D. (1991) J. Biol. Chem. 266, 15598–15601.
[36] Hormia, M., Lehto, V.-P. and Virtanen, I. (1984) Eur. J. Cell Biol. 33, 217–228.